

# Use of the polymerase chain reaction for the detection of *Chlamydia trachomatis* in clinical specimens and its comparison to commercially available tests

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## Abstract

A polymerase chain reaction (PCR) was developed to detect *Chlamydia trachomatis* in genital tract specimens. Two sets of primers for the PCR were used; one set amplifies a region of the plasmid present in all *C trachomatis* strains and the other amplifies a conserved region of the genome coding for the major outer membrane protein. The sensitivity of these PCRs were compared with each other, and with the sensitivities of antigen ELISA, *Clearview* and culture. Southern blotting and probing was used to increase sensitivity of detection.

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Keywords: chlamydia trachomatis; polymerase chain reaction

## Introduction

*Chlamydia trachomatis* is one of the most common human pathogens causing diseases that range from trachoma to female infertility. Diagnosis of the sexually transmitted strains is very important as the disease may go undiagnosed for long periods. Chlamydial infections can be asymptomatic. Recent studies in some countries have shown as many as 40% of adolescent women were infected.<sup>1</sup> It is these asymptomatic young women who are most likely to suffer long-term consequences of the infection.<sup>1</sup> It is therefore important to develop sensitive diagnostic methods to detect chlamydia in genital specimens for early treatment to be instituted.

Many tests are available for the detection of *C trachomatis*. These include direct immunofluorescence (DIF), antigen ELISA and culture identification, all of which vary vastly in technical requirements as well as sensitivity. Culture is recognised as the "Gold Standard" with which all other tests are compared. It is specific and if done correctly is sensitive. However, it is expensive and time consuming. A newer, more rapid test has been developed to accelerate diagnosis and a number of studies have already been performed to compare the sensitivity and ease of this test with culture and DIF.<sup>2,3</sup> This test which detects antigen in a solid phase and is marketed as *Clearview* was included in this study.

With the advent of the polymerase chain reaction (PCR),<sup>4</sup> a new diagnostic test is possible which has the potential to greatly increase

sensitivity of detection. We have developed two PCRs to detect *C trachomatis* in clinical specimens and have compared their sensitivity with each other as well as with culture, Antigen ELISA and *Clearview* using the L2 strain of *C trachomatis*. PCR products were also subjected to Southern blotting and probing to increase sensitivity of detection, and to confirm the nature of the PCR products.

## Materials and methods

*Clinical specimens* were obtained from a Family Planning Clinic where patients are routinely screened for chlamydial infection prior to voluntary terminations of pregnancy. These cervical samples are routinely tested for chlamydia by culture of swabs and direct immunofluorescence (DIF) on cervical smears. Antigen ELISA (IDEIA) is performed on samples that are unable to be cultured due to toxicity or contamination.

*Sensitivity of tests.* To compare sensitivities of each test, a strain of L2 was grown in HeLa 229 cells, harvested after 48 hours by three freeze thaw cycles, spun to remove cell debris and the supernatant containing chlamydia collected. This was serially diluted in sucrose phosphate-buffered transport medium (2SP) from  $10^{-0}$  to  $10^{-10}$  and a 200  $\mu$ l aliquot of each dilution tested by culture, antigen ELISA, *Clearview* and PCR. The PCR products were analysed by both agarose gel electrophoresis and Southern blotting.

*Culture* was done in HeLa 229 cells grown in LabTek 8-chambered slides in Medium 199 (M199) containing 10% foetal calf serum (FCS). A 200  $\mu$ l aliquot of either sample or purified L2 was inoculated into a single well, spun at 2,000 rpm at 37°C for 60 minutes and the medium changed after a further 60 minutes to M199 containing 1% FCS and 2  $\mu$ g/ml cyclohexamide. After 48-72 hours, the medium was removed and the monolayer fixed in methanol. The monolayers were then stained with the *Pathfinder Chlamydia Confirmation System* (Kallestad, Austin, Texas, USA) following the manufacturers instructions and scanned at 100 $\times$  using a Zeiss Fluorescent microscope. A positive control was included in each test run.

*Antigen ELISA* (IDEIA, Celltech) and *Clearview* (Oxoid) were performed following the manufacturers' instructions on 200  $\mu$ l aliquots of clinical samples or purified L2 organisms.

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## POLYMERASE CHAIN REACTION

(1) *Specimen preparation and DNA amplification.* A 200  $\mu$ l aliquot of sample was mixed with an equal volume of 100 mM Tris, 40 mM disodium-EDTA, 40% w/v sucrose, 1.4% w/v N-laurylsarcosine. Proteinase K was added to give a final concentration of 200  $\mu$ g/ml. Samples were mixed, incubated at 55°C for 15 minutes followed by 37°C for 45 minutes according to the method of McClenaghan *et al.*<sup>5</sup> DNA was purified using phenol-chloroform, precipitated overnight at -20°C in ethanol containing 3M Na Acetate then pelleted and resuspended in 20  $\mu$ l diethylpyrocarbonate (DEPC) treated water.

(2) *Reaction* 1  $\mu$ l of DNA was used in each 50  $\mu$ l PCR reaction containing 1  $\times$  Taq polymerase buffer (50 mM KCl, 10 mM Tris-HCl pH 8.8 at 25°C, 0.1% Triton-X 100), 2.5 U Taq polymerase (Promega) and nucleotides (Promega) to a final concentration of 10 mM [dATP, dCTP, dGTP and dTTP]. Primers were used at 50 pmol and were directed to; (a) The plasmid found in all *C. trachomatis* serovars<sup>6</sup> and consisted of the following: Primer 1A: 5'-GTT TAA GTG TTC CCA TCA TAA AAA CAT ATT C-3' Primer 1B: 5'-ATC CTT GTA TCC TGT TGG GAA GCC ATC AAA G-3' (b) A conserved region of the major outer membrane protein (MOMP): Primer 110; 5'-AGA GGT AAG AAT GAA AAA ACT CTT GAA ATC GG-3' Primer CT6<sup>7</sup>; 5'-TTT TCT AGA TTT CAT CTT GTT CAA (T/C)TG-3'.

Amplification was in a DNA Thermal Cycler (Perkin Elmer Cetus, Connecticut USA) using 40 cycles and optimal temperature and magnesium concentrations for each primer set. Optimal annealing temperature was 45°C for both reactions, and magnesium concentrations were 1.5 mM, and 2.0 mM for the MOMP and plasmid PCRs respectively. Cycling parameters were 94°C for 1 minute, 45°C for 1 minute and 72°C for 3 minutes.

(3) *Controls* DNA extracted from the L2 strain grown in HeLa cells was used in each PCR run as a positive control. A negative control of DNA extracted from HeLa cells only was also included.

(4) *Interference* To check the effect of mucus and blood present in diagnostic samples on the PCR, samples negative for *C. trachomatis* by culture, DIF and PCR were tested after addition of L2. These samples had also been found to be antibody negative when tested by a commercially available ELISA test (BioWhittaker Inc. Walkersville, MD USA).

(5) *Analysis of PCR product* PCR products were analysed on a 1% agarose gel which incorporated ethidium bromide. Products were viewed using a transilluminator and photographed.

(6) *Southern Blotting* PCR products were transferred to nitrocellulose using a standard Southern blotting method.<sup>8</sup> Briefly, after photographing, the gel was soaked in 0.25 M HCl for 15 minutes and rinsed twice in distilled water. The DNA gel was then denatured by soaking in 0.5 M NaOH, 1.5 M NaCl for 30 minutes and then again for another 30 minutes. The gel was again rinsed twice in distilled water then neutralised by soaking in 1 M ammonium acetate, 20 mM NaOH for two 30 minute periods. After rinsing in distilled water twice more the gel was transferred to a nitrocellulose membrane (Schleicher and Schuell) using standard techniques.

(7) *Probe preparation* Digoxigenin (DIG) (Boehringer-Mannheim) labelled probes that were complementary to the amplified products were prepared by PCR based on the method of Lion and Haas.<sup>9</sup> Briefly, DIG-dUTP was incorporated into a PCR reaction mix with control L2 DNA as template and primers to the *C. trachomatis* plasmid or MOMP. Amplification was for 40 cycles as described above.

(8) *Probe analysis of PCR products* After Southern blotting the nitrocellulose was subjected to 3 min of UV light to bind irreversibly the transferred DNA. The nitrocellulose was then probed using the newly prepared DIG-labelled probes following the Boehringer Mannheim Nucleic Acid Detection Kit method.

## Results

Table 1 compares the ability of all methods to detect purified cell culture L2 chlamydia. This table shows that culture is 10 times more sensitive than IDEIA and 100 times more sensitive than *Clearview*.

The PCR reactions showed a variation in sensitivity. The MOMP primers PCR, as judged by visible agarose gel bands, was less sensitive than culture. In comparison the plasmid PCR showed a 10 fold increase in sensitivity when compared with culture.

The deficiencies of the agarose gel system were highlighted when probing was used to detect PCR products. This showed that PCR with either set of primers was more sensitive than culture and indicated that using plasmid primers and sensitive product detection increased detection by 1000 fold. The figure illustrates an agarose gel and subsequent southern blotting of the MOMP PCR performed on dilutions of cell culture grown L2.

Cervical samples that had been found to be negative for either viable chlamydia organisms, chlamydial antigens or antibody were seeded with small amounts of the L2 organism. Aliquots were tested both before and after seeding by plasmid PCR. In all 12 samples were tested. Results showed there was no interference by any agents present. All samples were negative by PCR when tested before seeding and all 12 samples gave a positive PCR test after seeding.

Table 1 Comparison of sensitivities of PCR versus commercially available chlamydia tests

Test	Dilution of L2										
	10 <sup>0</sup>	10 <sup>-1</sup>	10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>	10 <sup>-6</sup>	10 <sup>-7</sup>	10 <sup>-8</sup>	10 <sup>-9</sup>	10 <sup>-10</sup>
IDEIA	+	+	+	+	+/-	-	-	-	-	-	-
Culture	+	+	+	+	+	+	+	+	+	+	+
Clearview	+	+	+	+	-	-	-	-	-	-	-
PCR-MOMP*	+	+	+	+	+	-	-	-	-	-	-
PCR-Plasmid*	+	+	+	+	+	+	+	-	-	-	-
Probe-MOMP†	+	+	+	+	+	+	+	-	-	-	-
Probe-Plasmid†	+	+	+	+	+	+	+	+	+	-	-

\*product analysed by agarose gel electrophoresis

†product analysed by DIG-labelled probe

Fig: Analysis of MOMP PCR by agarose gel electrophoresis (1A) and southern blotting (1B). Lane 1; mixture of  $\lambda$ /HindIII and pUC19/HpaII DNA markers. Sizes are in kilobase pairs. Lane 2–8; L2 DNA serial dilutions  $10^0$ – $10^{-6}$ . Lane 9; Positive control.

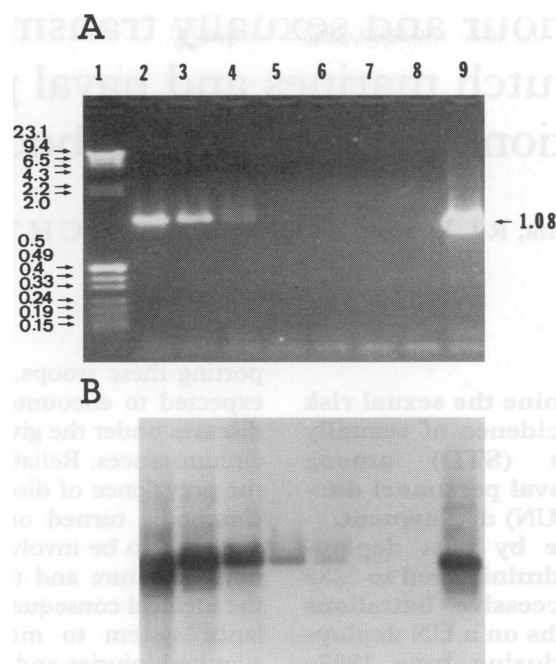


Table 2 Comparison of PCR using plasmid primers and culture results of diagnostic samples

	Culture POS	Culture NEG	
PCR POS	45	4	49
PCR NEG	5	50	55
Total:	50	54	104

A series of clinical samples were tested by both PCR and culture. The results of these are shown in table 2. The table shows that when compared with culture, PCR has a sensitivity of 90% and a specificity of 93%.

### Discussion

The *Clearview* test was the easiest test to perform with no specialised equipment apart from a water bath required. Results are obtained within 30 minutes of commencing the test but the sensitivity is greatly reduced when compared with other tests. It was slightly less sensitive than the more time consuming antigen ELISA but at least 100 fold less sensitive than culture and PCR.

Although the primers chosen had a relatively low GC content, the efficiency of the reaction was still such that the sensitivity of detection was equivalent to that of culture. Although the MOMP primers were directed to a conserved region, there is a one base variation between some *C trachomatis* strains. The "wobble" in primer CT6 was therefore included so all human *C trachomatis* strains could be amplified by this primer set.

In the PCR test plasmid primers were found to be more sensitive than the MOMP primers. This increased sensitivity of the plasmid primers is expected as there are at least 10 copies of the plasmid in each organism. The plasmid PCR was found to be 10-fold more sensitive than culture, and both the plasmid and MOMP PCR's sensitivity were increased by the use of probes. This finding is in agreement with that of Naher *et al*<sup>10</sup> who report a 100 fold increase in sensitivity with PCR when compared with culture. Others<sup>11 12</sup> have found

PCR to be of comparable sensitivity and specificity to culture.

No interference was found when negative diagnostic samples were seeded with L2. The samples chosen contained mucus and some had small amounts of blood present, both substances which can inhibit the PCR.

PCR does have other advantages over culture in that the result is obtained within two days without a compromise in sensitivity. The strict transport conditions required for culture of these organisms are not required for reliable detection by PCR.

Many commercially available kits are currently used to determine the presence of chlamydial infection. The most sensitive method until now has been culture but this is not available in all diagnostic laboratories and has been confined mainly to services that also provide viral diagnosis by culture. Although PCR requires specialised techniques, these are more achievable for smaller laboratories than cell culture. If the sensitivity is equivalent to or better than that of culture, it appears that PCR could be used routinely in chlamydia diagnosis.

This laboratory has successfully applied this new technique to diagnostic samples. Results on a number of samples have shown that the sensitivity and specificity of the PCR is 90% and 93% respectively. This shows good correlation between the PCR and the gold standard, culture. However, PCR does not require the same degree of care and attention in maintaining organism viability and is particularly useful in toxic or contaminated specimens. Problems with possible contamination leading to false positive results may limit the usefulness of PCR in routine diagnosis but this could be overcome by the use of a confirmatory test such as DIF or culture. PCR should therefore have a role in routine diagnosis and in screening selected populations.

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